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Role of organic and ceramic biomaterials on bone healing and regeneration: An experimental study with significant value in translational tissue engineering and regenerative medicine

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ABSTRACT

Objective(s): We investigated the role of various biomaterials on cell viability and in healing of an experimentally induced femoral bone hole model in rats.

Materials and Methods: Cell viability and cytotoxicity of gelatin (Gel; 50 µg/µl), chitosan (Chi; 20 µg/µl), hydroxyapatite (HA; 50 µg/µl), nanohydroxyapatite (nHA; 10 µg/µl), three-calcium phosphate (TCP; 50 µg/µl) and strontium carbonate (Sr; 10 µg/µl) were evaluated on hADSCs via MTT assay. *In vivo* femoral drill-bone hole model was produced in rats that were either left untreated or treated with autograft, Gel, Chi, HA, nHA, TCP and Sr, respectively. The animals were euthanized after 30 days. Their bone holes were evaluated by gross-pathology, histopathology, SEM and radiography. Also, their dry matter, bone ash and mineral density were measured.

Results: Both the Gel and Chi showed cytotoxicity, while nHA had no role on cytotoxicity and cell-viability. All the HA, TCP and Sr significantly improved cell viability when compared to controls (P<0.05). Both the Gel and Chi had no role on osteoconduction and osteoinduction. Compared to HA, nHA showed superior role in increasing new bone formation, mineral density and ash (P<0.05). In contrast to HA and nHA, both the TCP and Sr showed superior morphological, radiographical and biochemical properties on bone healing (P<0.05). TCP and Sr showed the most effective osteoconduction and osteoinduction, respectively. In the Sr group, the most mature type of osteons formed.

Conclusion: Various biomaterials have different *in vivo* efficacy during bone regeneration. TCP was found to be the best material for osteoconduction and Sr for osteoinduction.

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Introduction

Bone injuries have a great incidence worldwide (1-3). Autografts and allografts are routinely used to reconstruct bone defects and to induce both osteoconduction and osteoinduction in order to prevent non-unions particularly in those injuries that are engaged with significant bone loss (1, 4). However, using auto- and allo- grafts have considerable limitations (1, 5). Bone tissue engineering and regenerative medicine have been introduced with the aim of providing alternative options to classic strategies (1-3). There is no doubt that bone scaffolds have considerable value in bone reconstructive surgery and also bone regeneration and repair (5, 6). Many bone scaffolds have been designed, fabricated and investigated for their role during bone regeneration. Since a desirable scaffold for bone tissue engineering should have porous structure, fabrication of a desirable bone scaffold is generally difficult. So far, various biomaterials with different formulations have been used to produce bone scaffolds (5, 7, 8). In this regard, material selection has a great role for designing an ideal scaffold. It has been extensively shown that the most beneficial effects of bone scaffolds during bone regeneration is at least in part due to the biomaterials that are used to fabricate such scaffolds (7, 9, 10).

Biomaterials can be divided into two major categories including natural (organic vs. inorganic), and synthetic (polymeric vs. ceramic) (1, 9, 11). In designing a suitable tissue engineered-based strategy, today we know that materials selection should mimic the native tissue microenvironment in order to facilitate bone regeneration (10, 12, 13). Although synthetic polymeric materials such as polylactic acid (PLA), poly-L-lactic acid (PLLA), poly lactic-co-glycolic acid (PLGA), polydioxanone (PDS) and polycaprolactone (PCL) have been the focus of many studies due to their excellent controllable biodegradation and drug delivery properties, the fact that such biomaterials have low bioactivity and healing efficacy during bone regeneration has limited their translational value these days (9, 13). To mimic the native microenvironment, it should be remembered that bone has been made up of organic and inorganic materials (10, 14). Thus, the natural biomaterials such as collagen, gelatin (Gel), chitosan (Chi), elastin and fibrin may be able to mimic organic microenvironment of the bone (9, 10). On the other hand, most of the bones have been made up of inorganic and ceramic materials, and the amount of hydroxyapatite (HA) in bone is high (10). Thus, calcium phosphorous crystals such as HA and three calcium

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phosphate (TCP) and also strontium (Sr) salts may mimic the bone inorganic part. Due to the close chemical similarity of synthetic HA, TCP and Sr to the natural form found in the inorganic component of the bone matrix, they may be a good candidate as an alternative to the natural source (9, 10, 15).

Gel is a hydrolyzed form of collagen that has been extensively used for cell culture applications and also fabricating bone scaffolds in combination with other compounds (16). Gel has been suggested to be biocompatible, biodegradable and safe biomaterial that can be easily used in bone tissue engineering (6, 14, 17). In addition, Chi is a natural structural polysaccharide and has many beneficial effects during cell culture and scaffold fabrication (14, 18, 19). For instance, incorporating Chi within bone scaffolds increases scaffold porosity (8, 18). According to the literature, both the Gel and Chi enhance cell migration, attachment, proliferation, and maturation particularly at *in vitro* level. In addition, these two biomaterials have been extensively used in many researches and are popular biomaterials these days (19).

Based on the current literature, HA, TCP and Sr salts have been extensively used for various bone tissue engineering applications (10, 20). Calcium phosphorous crystals such as HA and TCP have been suggested to mimic inorganic part of bone microenvironment and are cytocompatible *in vitro*, but more importantly they are biocompatible and biodegradable *in vivo* (18, 20). Both the HA and TCP have been used for scaffold fabrication with beneficial osteoconductive properties at *in vivo* level (14, 18). Recently and with introduction of nanomedicine to bone tissue engineering, nanohydroxyapatite (nHA) has also been introduced with more beneficial results than microstructured HA (12, 16, 21). Thus, it has been suggested that size and shape of the biomaterials have also effect on the final *in vivo* behavior of a material (12). Sr salts such as Sr acetate and Sr carbonate have also recently been introduced in the literature and many bone scaffolds using a variable combinations of HA, TCP and Sr have been fabricated and used both *in vitro* and *in vivo* (14, 20). Sr ion has been suggested to be osteoinductive compound, which is an important characteristic for bone regeneration (20, 22).

Despite passing decades in bone research, we currently know the most important biomaterials for bone repair (17). However, most of these biomaterials have only been investigated at either *in vitro* or *in vivo* levels and limited data exist showing their *in vitro* and *in vivo* efficacy in comparison with each other in order to provide a guideline for scaffold designers to fabricate the most effective formulation for bone regeneration (17, 20). Therefore, this study was designed to determine the *in vitro* cytotoxicity and cell viability effects and to investigate the role of various popular biomaterials on healing of an experimentally induced femoral bone hole model in rats. We hypothesized that because a great background exists showing the cytocompatibility and biocompatibility of the Gel, Chi, HA, nHA, TCP and Sr, probably all these biomaterials not only should have no cytotoxicity *in vitro* but also should be cytocompatible *in vitro* and biocompatible *in vivo* (20). Because Gel and Chi are organic compounds and hove no significant bone signaling properties, they should have least osteoinductive properties (20). However, based on the literature and because these two latter biomaterials have been shown to improve and enhance cell migration and proliferation, they may have osteoconductive properties *in vivo* (17). HA and TCP should improve osteoconduction, and least osteoinduction should be observed after application of them in the bone holes. In addition, because nHA has small particle size than HA, it may show superior interactions with the regenerative bone, so that it may have some osteoinductive properties (16). Because Sr has been shown to have considerable effects on bone signaling pathways and differentiate the stem cells to osteoblasts *in vitro*, its *in vivo* application should provide acceptable osteoinduction (20).

Materials and Methods

Materials

All the type B Gel, medium molecular weight Chi, TCP and strontium carbonate (Sr) were purchased from Merck (Germany). HA was purchased from Sigma Aldrich (Germany). Nano HA with the particle size less than 100 nm was purchased from Hayan Yazd Nano-Chemistry (Research Based Company, Pazhoohesh Fanavaran Yazd, Yazd, Iran). The mentioned biomaterials were purchased as powder so that their structure was characterized under scanning electron microscopy (SEM) using gold-coated samples. All other materials and reagents were purchased from Sigma Aldrich unless specified.

Cell cytotoxicity and viability assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was used for the quantitative determination of cytotoxicity and cell viability assay (23). The experiments were carried out in 96-well plates according to the cell proliferation kit protocol (Roche GmbH, Mannheim, Germany). In the MTT test, tetrazolium salts were transformed by active enzymes of the cells into intracellular formazan deposits, cells were incubated for 4 hr with the tetrazolium salts, after this incubation time the purple formazan salts formed became soluble. Absorbance was determined at 570/630 nm with an ELISA reader. Human subcutaneous adipose tissue samples were obtained after liposuction surgery from the abdomen region and donated after informed consent of the patients from Rasool Akram hospital in Tehran, Iran. Adipose derived mesenchymal stem cells (ADSCs) were derived from adipose tissue as described previously (24). Cells were characterized by flowcytometric analysis of the expression of cell surface markers according to a previously published method (25). The hADSCs were seeded at a concentration of 5000 cells/well in 96-well plates in order to obtain approximately 40% confluence. Cells were allowed to attach overnight in DMEM medium supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin. We used direct exposure system. Before we made a comparison between different biomaterials, we used a pilot MTT assay to evaluate the most effective concentration of each biomaterial on cell viability and cell cytotoxicity. The biomaterials (as powder) were suspended in DMEM to provide various concentrations. Thus, for each biomaterial, several

concentrations were prepared including 1 µg/µl, 2 µg/µl, 5 µg/µl, 10 µg/µl, 20 µg/µl, 30 µg/µl, 40 µg/µl, 50 µg/µl, 60 µg/µl, 70 µg/µl, 80 µg/µl, 90 µg/µl and 100 µg/µl. We selected the maximum concentration for each biomaterial so that in that concentration, the cell viability and cytotoxicity would not significantly alter when compared to the lower concentrations. For comparison between desired concentrations of various biomaterials, Gel (50 µg/µl), Chi (20 µg/µl), HA (50 µg/µl), nHA (10 µg/µl), TCP (50 µg/µl), and Sr (10 µg/µl) were individually sterilized under UV irradiation for 20 minutes at triplicate, and then suspended in a culture medium and stirred at 500 rpm for 5 sec. The final medium was evaluated under light microscopy for validating homogenous dispersion of the biomaterials. After validating, 50 µl of the suspension was transferred into each of the well plates containing 100 µl of culture medium+5000 attached ADSCs. Thus, the stem cells were in direct exposure of the biomaterials. The MTT assay was performed after 24, 48 and 72 hr after direct exposure to the biomaterials (n=3 for each biomaterial at each time point) (23).

Scanning electron microscopy of the biomaterials

To study the morphology of the biomaterials at ultrastructural level, the biomaterials (Gel, Chi, HA, nHA, TCP and Sr) were sprayed on the paper that was freshly soaked with slow drying glue. Then, the glue was left to dry at room temperature. The samples were treated with hexa-methyl disilazane and osmium tetra-oxide, respectively and then gold coated under vacuumed condition. Under SEM (Philips, Eindhoven, Netherlands), ultra-micrographs were provided using variable Kilovolts (KVs) and magnifications (6).

In vivo experiment

Ethics

All animals received humane care in compliance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1985). The study was approved by the local Ethics Committee of our Research Center.

Experiment

A number of 40 femoral bones in 20 skeletally and sexually mature Wistar rats (weight = 200-250 grams) were randomly divided into 8 equal groups (each had 5 femurs). The animals were first premedicated by intramuscular administration of Acepromazine maleate 2% and then anesthetized via intra-peritoneal injection of Ketamine HCl 10% (60 mg/kg) and Xylazine 2% (20 mg/kg). After clipping the hairs over the hind limbs and under aseptic condition, a dorsolateral skin incision was made over the femoral shaft and the rectus femoris and vastus lateralis muscles were dissected to expose the lateral cortex of the femoral shaft. In each femur, 5 holes (each had 3 millimeters in diameter) were surgically created in the lateral cortex of the femoral shaft using electrical micro drill. The drilling was performed at 150 rpm and under saline irrigation to prevent thermal necrosis. These numbers of holes were designed to 1) increase the samples size and 2) increase the reliability of the test. In the negative control group, all the 5 holes in each femur (n=5; totally 25 holes) were left untreated. In the autograft group, the holes (n=25) were filled completely with autograft powder (the powder was harvested and collected from the rat femoral cortex after drilling femoral cortex).

In the rest of the groups (test groups; each had 5 femurs and totally 25 bone holes), the holes were treated (filled completely) with Gel, Chi, HA, nHA, TCP and Sr powders, respectively. All the Gel, Chi, HA, nHA, TCP and Sr we used in the present experiment were provided commercially as powder by the companies (see above). The holes were filled with powders to a level that they did not have more space to fill it with material powder. Then, we let the blood that came out of the femoral cortex incorporate with the filling powder waiting for 10 minutes to ensure that the blood clotting is done and the powder of various materials are stable in the bone holes. Then, the muscles, subcutaneous tissues and the skin over the lesion were approximated in a routine fashion. Post-operative analgesia and antibiotic were provided by subcutaneous administration of 4 mg/kg Tramadol and enrofloxacin (5 mg/kg) for three days, respectively.

Euthanasia

Thirty days after bone injury, the animals were anesthetized via Ketamine HCl and Xylazine HCl as described before. Under anesthesia, the animals were euthanatized in the CO_2 chamber. The mean euthanasia time was bellow 15 seconds.

Gross pathology

Immediately after euthanasia, the femoral bones (n=5 in each group) were harvested and evaluated for gross pathology. In gross pathology, the holes (5 holes in each femur; number of femur in each group=5) were divided into five groups including: 1) the holes that were completely open and did not fill with any new tissue, 2) the holes that were partially filled with soft tissue, 3) the holes that were completely filled with soft tissue, 4) the holes that were partially filled with hard tissue and 5) the holes that were completely filled with hard tissue and closed. For each group, number of holes was individually counted based on the above categories. In addition, the injured healing bone holes were evaluated for their pathologic appearance including hyperemia, necrosis, presence or absence of biomaterials, hypertrophy, metaplasia, and atrophy (6).

Plain radiography

All the femurs were subjected to digital radiography and lateral radiographs were provided using 50 KV and 5 mA. In radiographs, the bone holes were evaluated for determining density of new bone formation in each hole, which was measured by Image J software (NIH, CA).

Bone densitometry

Dual-energy X-ray absorptiometry (DEXA, XR-36, Norland, Connecticut, USA) was used to measure the bone mineral density (BMD) of a 3×3 mm^2 area.

Sample collection

In each group, 12 femoral bone holes were randomly

selected and used for histopathology and SEM and the rest (n= 13 femoral bone holes) were used to determine and measure dry matter content and bone ash.

Histopathology and histomorphometry

The samples were initially fixed with buffered formalin 10%. The fixative solution was changed after 24 hr to prevent formalin pigmentation. Then, the samples were decalcified in formic acid 5% for 10 days, dehydrated in a graded series of ethanol, cleared in xylene, embedded within paraffin wax, serially sectioned (n=3) at 4 µm longitudinally and transversely, stained with hematoxylin and eosin and also with Masson three chrome and studied by a light microscope (Olympus, Tokyo, Japan). The employing magnifications were ranging from 40X to 1000X. Under light microscopy, digital micrographs were provided for each section of each bone hole from three different microscopic fields. The micrographs were transferred to the computer software (Adobe Photoshop, and Image J, NIH, both from CA) for digital morphometry and cell counting. Different cells including fibroblast, fibrocyte, chondroblast, chondrocyte, osteoblast, osteocyte, neutrophil, lymphocyte, macrophage, and osteoclast were counted from micrographs provided at 400X (6). Primary and secondary osteons were counted at ×100 and the density of the new bone formation was calculated at ×40. Before cell counting, the cells were differentiated from each other based on the basic principles of pathology according to a previously published method (6, 26-28). In addition, transverse diameter and area of the osteons were measured by Image J software (27). Other pathologic features of the injured healing bone holes were also described and reported when necessary.

Scanning electron microscopy

The samples were prepared as described before (26, 29). Under SEM, morphology of the samples was studied using different magnifications ranging from ×50 to ×250000. To determine chemical composition of the structures and to define different structures from each other, energy dispersive x-ray spectroscopy (EDX) was used when necessary. Under SEM, the ultrastructure of the osteons, haversian canals, cellular structures, collagen fibers and bone crystals were studied.

Dry matter content and bone ash

Dry matter content was calculated using the following equation: Percentage dry matter content=(dry weight (DW)/wet weight (WW)×100 (30). For determining bone ash, the dehydrated samples were ashed in a muffle furnace overnight at 600 ˚C, and weighed again after ashing. Percentage of ash was calculated as: Ash weight/DW×100.

Statistical analysis

The results were expressed as either % or mean ± standard deviation. Differences between the measured values were statistically tested using One-way ANOVA with its subsequent *post hoc* Tukey tests. The correlation test was performed by ''Pearson's correlation test''. All the tests were performed using the statistical package for Social Sciences, version 22, for Windows (IBM Corp. Armonk, NY.). Values lower than < 0.05 were considered significant.

Inter-tester and intra-tester reliability

The investigators who undertook the measurements and analyses of the results were unaware of the experimental design and grouping details. Each evaluation and measurement was performed at triplicate and the mean of the measured values were reported. For radiologic, gross pathologic, histopathologic and SEM evaluations, three expert scientists evaluated the samples and reported their results. No significant differences were observed between the results of the individuals ($P>0.05$).

Criteria for evaluating osteoconduction, osteoinduction and osteogenesis

Osteoconduction is a characteristic whereby the graft acts as a permanent and resorbable scaffold, mechanically supporting ingrowth of vessels and new bone from the borders of the defect into and onto its surfaces (1). The number of vessels and osteoblasts together with orientation of the newly formed bony tissue from the edges of the bone-hole into the center of the hole under light microscopy, filled bone holes at radiology, distribution of Calcium and phosphorus elements at EDX and density of Calcium at BMD were considered as criteria for osteoconduction. Osteoinduction is the capability of the graft materials to induce formation of the bone-forming cells via differentiation of multipotent mesenchymal stem cells of the surrounding host tissues to produce osteoprogenitor cells followed by development of osteoblasts (1). The higher number of osteoblasts, osteoclasts, osteocytes, and macrophages compared to the number of neutrophils, lymphocytes, giant cells, fibroblasts, fibrocytes, chondroblasts and chondrocytes was considered as the criteria for osteoinduction that was defined under light microscopy of the *in vivo* specimens. Osteogenesis is the capacity to produce new bone by the osteoblasts through differentiation of osteoprogenitor cells either present in the recipient bone or coming from the graft material (1). The number and quality (e.g. their transverse diameter) of primary and secondary osteons and their formations under SEM and light microscopy were considered as criteria for osteogenesis.

Results

***In vitro* evaluations**

Cytotoxic effect of biomaterials and their cell viability

Cytotoxicity of the biomaterials was determined based on their lower optical density measured by MTT assay from the negative control. Both the Gel and Chi showed toxic effects on hADSCs at all-time points including 24, 48 ad 72 hr after cell exposure and the optical density measured for these two biomaterials were significantly lower than control and other biomaterials ($P=0.001$ for all the comparisons). Compared to Chi, Gel had less toxic effects at 24 hr but showed higher toxic effects at 48 and 72 hr of cell exposure ($P=0.001$ for all the comparisons). Nano HA showed no significant toxic effects after cell exposure when compared to the negative control group at all of the time points ($P>0.05$). None of the HA, TCP

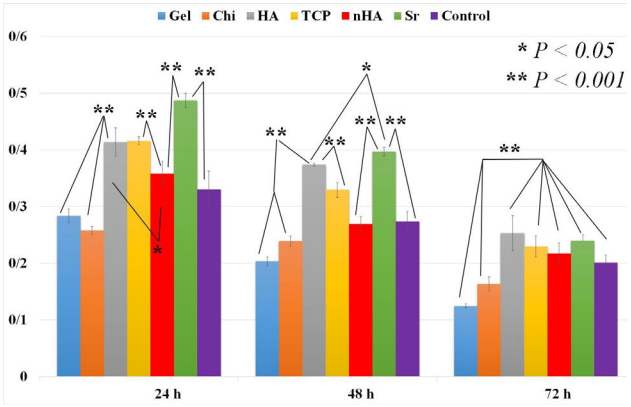

Figure 1. Cell viability and cytotoxicity were determined by MTT assay
In the MTT test, tetrazolium salts were transformed by active enzymes of the ADSCs into intracellular formazan deposits, cells were incubated for 4 hr with the tetrazolium salts, after this incubation time the purple formazan salts formed became soluble. Absorbance was determined at 570/630 nm with an ELISA reader. Cytotoxicity and cell viability effects of various biomaterials were evaluated on hADSCs at three different time point including 24, 48 and 72 hours after cell seeding on the well plates. Note that Gel and Chi significantly reduced cell viability and were cytotoxic for the ADSCs at all-time points (P<0.05), while nHA had no significant cell cytotoxicity and also did not increase cell viability (P>0.05). In contrast, HA, Sr, and TCP significantly improved cell viability at all-time points when compared to the controls (P<0.05). ADSCs: Adipose derived mesenchymal stem cells, Gel: Gelatin, Chi: Chitosan, HA: Hydroxyapatite, nHA: Nanohydroxyapatite, TCP: Three calcium phosphate, Sr: Strontium

and Sr showed cytotoxic effects at all-time points when compared to the control group (Figure 1).

Cell viability of the biomaterials was determined based on their higher optical density measured by MTT assay from the negative control. Both the Gel and Chi had no beneficial effects on cell viability at all-time points. Nano HA slightly increased cell viability at 24 hr of cell exposure but the differences were not statistically significant with the control group ($P>0.05$). All the HA, TCP and Sr significantly increased cell viability when compared to the control, Gel and Chi groups at all of the time points (P=0.001 for all the comparisons). At 24 and 48 hr after cell exposure, Sr showed the highest optical density and significantly increased cell viability compared to HA and TCP (P=0.001 for all). In addition, there were no significant differences for cell viability (optical density) of the TCP and HA at 24 hr (P>0.05) but after 48 hr, HA showed superior value when compared to TCP (P=0.001). At 72 hr after cell exposure, there were no significant differences in term of cell viability between Sr, nHA, HA, and TCP (P>0.05) (Figure 1).

Ultrastructure of the biomaterials

The ultrastructures of the purchased biomaterials were studied under SEM. The Gel particles were micro-structured and spherical with transverse diameter ranging from 2 to 4.8 µm with average diameter of 2.76±0.51 µm. The Chi particles were micro-structured spherical particles with a wide variety in diameter ranging from 319 nm to 4.5 µm and average of 2.91±1.14 µm. Surfaces of the Gel and Chi particles were smooth; however, the Chi microparticles were smoother and rounder. Similar morphology regarding crystalline structure was observed in both the HA and nHA. However, they were different in the particle size. In the HA group, the particles had transverse diameter ranging from 2.96 to 8.82 µm and average of 5.84±0.79 µm, while in the nHA group, the particles had transverse diameter ranging from 35 to 98 nm and average of 76.32±12.95 nm. TCP particles were star-shape with very wide range of largest diameter ranging from 4.52 to 35.97 µm and average of 14.86±7.82 µm. Sr particles were square-shape with very smooth surfaces that had nanometric thickness with largest diameter ranging from 1.83 to 3.28 µm and average of 2.53±0.31 µm (Figure 2).

In vivo evaluations

Clinical evaluations

None of the animals died during the course of the experiment. The animals had good appetite and physical activity and none of the wounds became infected.

Gross pathology and radiology

No signs of necrosis, inflammation, hyperemia and tumor were observed in any of the holes and all the

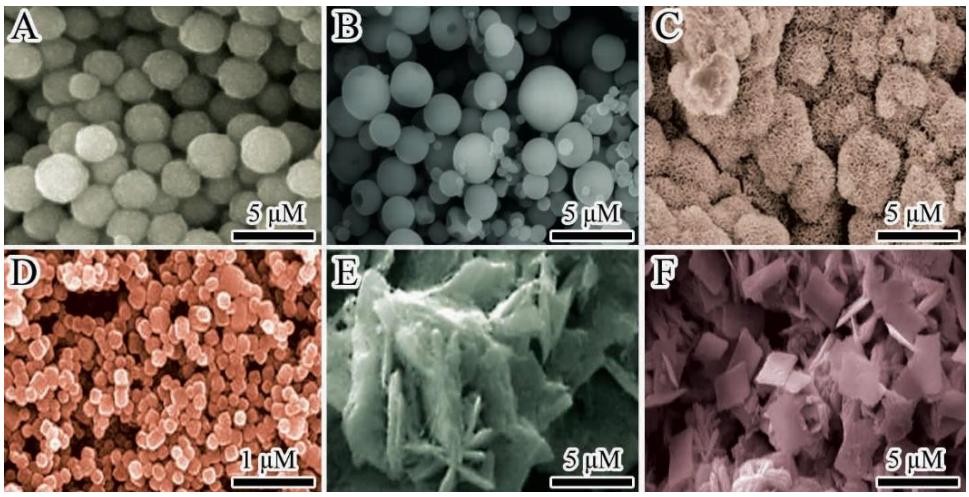

Figure 2. Scanning electron micrographs of various biomaterial powders
Note that various biomaterials have different shapes ultrastructurally and this could be potentially responsible for their *in vivo* efficacy during bone regeneration

Table 1. Percentages of bone holes in each group that were either closed or open after thirty days of application of various biomaterials

Group	Completely closed with bone	Partially closed with bone	Completely closed with soft tissue	Partially closed with soft tissue	Open
Untreated	0	0	0	20	80
Autograft	52	36	8	4	0
Gel	0	0	12	8	80
Chi	0	16	10	4	70
Hydroxyapatite	40	44	0	0	16
Three calcium phosphate	100	0	0	0	0
Nanohydroxyapatite	84	4	16	0	0
Strontium carbonate	80	20	0	0	0

Number of bone holes in each group=25

biomaterials were completely resorbed after 30 days of bone injury.

Thirty days after bone injury, 80% of the untreated bone holes were still open and the rest were partially closed with soft tissue. In the Gel group, same percentage of the holes was entirely open and 8 and 12% of the holes were partially or totally closed with soft tissue, respectively. In the Chi group, 70% of the bone holes were open, 4 and 10% of the holes were partially or totally closed with soft tissue and 16% of the holes were partially closed with hard tissue. In the untreated holes and also in the Gel and Chi groups, actually none of the holes were totally closed with bone. In the autograft, HA, nHA, TCP and Sr groups, none of the holes were totally open. Four percent of the autograft holes were partially closed with soft tissue, while none of the holes of the HA, nHA, TCP and Sr closed with soft tissue. In the nHA and autograft group, 16 and 8% of the holes were totally closed with soft tissue, respectively, and none of the holes in the HA, TCP and Sr were closed with soft tissue. In the HA, autograft, Sr and nHA, 44, 36, 20 and 4% of the bone holes were partially closed with bone, respectively. In comparison with other groups, 100% of the TCP-treated holes were totally closed with bone, while in the nHA, Sr, autograft and HA groups, 84, 80, 52 and 40% of the holes were totally closed with bone at this stage, respectively (Table 1; Figure 3).

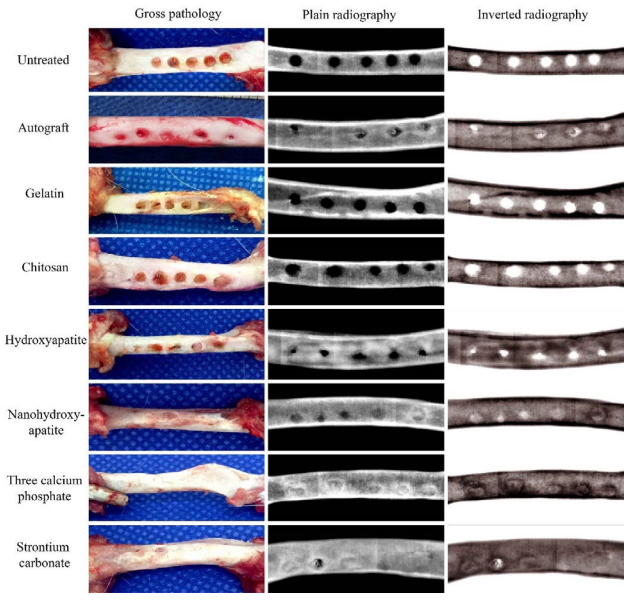

Figure 3. Gross pathology and plain radiography of the injured healing femoral bone holes, 30 days after bone injury
Note that most of the bone holes in the untreated group and also in those that were treated with Gel and Chi are open at this stage. In contrast, in the HA-treated bone holes, some of the holes closed, while some others are open. In addition, in those groups that were either treated with nHA, TCP and Sr completely, the bone holes closed after 30 days of bone injury. Gel: Gelatin, Chi: Chitosan, HA: Hydroxyapatite, nHA: Nanohydroxyapatite, TCP: Three calcium phosphate, Sr: Strontium

Histopathology

Thirty days after bone injury, the Gel and Chi groups showed significantly lower fibroblasts and higher fibrocytes than the untreated group (P=0.001 for all). In addition, the Gel group showed significantly higher fibrocytes compared to Chi group (P=0.001). The Chi group also showed significantly higher chondroblasts, chondrocytes, osteoblasts and osteocytes, primary osteons and also neutrophils and osteoclasts when compared to the untreated and Gel groups (P<0.05 for all). Compared to the Gel, Chi significantly increased the number of lymphocytes (P=0.001). At this stage, HA significantly increased osteoblasts, osteocytes, primary and secondary osteons compared to untreated, Gel and Chi groups (P=0.001 for all). Compared to autograft group, the HA group showed significantly higher primary osteons (P=0.001). Compared to HA, the nHA showed significantly higher osteoblasts and osteocytes and also secondary osteons and osteoclasts after 30 days of injury (P=0.001 for all). At this stage, also the nHA group showed significantly higher chondroblast, chondrocyte, osteocyte and also primary and secondary osteons when compared to the autograft and untreated groups (P=0.001 for all). Compared to nHA, TCP had significantly higher secondary osteons at this stage (P=0.001). In addition, the TCP-treated group showed significantly higher number of osteocytes and secondary osteons compared to autograft (P=0.001) and also had higher number of osteoblast and osteocytes and also primary and secondary osteons compared to untreated, Gel and Chi groups (P=0.001 for all). The Sr group showed significantly lower number of fibrocytes and fibroblasts and also higher number of osteocytes

Table 2. Histopathologic features of the healed bone holes after 30 days of injury

	Untreated	Autograft	Gel	Chi	Hydroxyapatite	Nano-hydroxyapatite	Three-calcium phosphate	Strontium carbonate
Fibroblast	61.20±5.89	5.60±2.70	22.40±7.99	18.80±3.27	15.20±1.64	12.40±4.77	7.40±2.30	0
Fibrocyte	7.60±2.30	3.00±1.58	42.20±4.81	21.00±4.00	23.20±3.83	10.00±5.24	7.60±2.30	0
Chondroblast	0	2.40±1.14	2.20±1.92	13.00±3.60	5.40±1.14	6.80±2.58	5.00±2.91	1.80±1.30
Chondrocyte	0	2.00±0.70	0.80±0.83	5.80±1.92	3.60±1.81	8.60±2.07	5.40±2.07	3.60±1.67
Osteoblast	0	15.80±3.03	0	4.80±1.78	10.20±2.16	19.40±5.31	9.20±1.92	4.00±2.91
Osteocyte	0	15.40±1.67	0	0.20±0.44	13.20±2.16	25.80±6.37	36.40±6.87	67.80±15.70
Primary osteon	0	3.80±1.30	0	0.80±0.83	14.20±3.27	18.40±1.81	4.20±1.48	3.40±1.81
Secondary osteon	0	0.60±0.55	0	0	0.80±0.84	5.20±1.92	20.40±2.60	19.60±3.50
Neutrophil	4.20±1.48	0.80±0.84	3.40±1.14	9.00±1.73	0.40±0.54	0.40±0.54	0.60±0.54	0
Lymphocyte	2.80±1.30	0.80±0.44	1.00±0.71	6.60±1.51	0.40±0.54	0.40±0.54	0.20±0.44	0
Macrophage	2.60±0.89	0.80±0.83	0.80±0.83	5.00±2.23	0.60±0.54	0.60±0.54	3.40±1.14	0.40±0.54
Osteoclast	0	3.80±0.83	0	0.40±0.54	0.60±0.54	4.20±3.03	0.40±0.54	2.00±1.22

The results were reported as Mean±standard deviation. Number of samples in each group=25, number of tissue sections in each sample = 3, number of histopathologic fields in each tissue section=3. Totally 225 histopathologic fields were used to count various cell types and osteons in each group. One-way ANOVA with its subsequent post hoc Tukey tests were used to statistically analyze significant differences between the groups. A value of<0.05 was considered statistically significant. Cells were counted at magnification=×400 and osteons were counted at magnification=×100

compared to untreated, autograft, Gel, Chi, HA, nHA, and TCP groups after 30 days of bone injury (*P*=0.001 for all). In comparison between the HA, nHA, TCP and Sr groups, nHA showed significantly highest number of osteoblasts (*P*=0.001 for all), Sr showed the highest number of osteocytes (*P*=0.001 for all), nHA showed the highest number of primary osteons (*P*=0.001, except for the HA group), TCP and Sr showed the highest number of secondary osteons (*P*=0.001 except for TCP vs. Sr) and Sr also showed least number of neutrophils and lymphocytes at this stage of bone regeneration (Table 2).

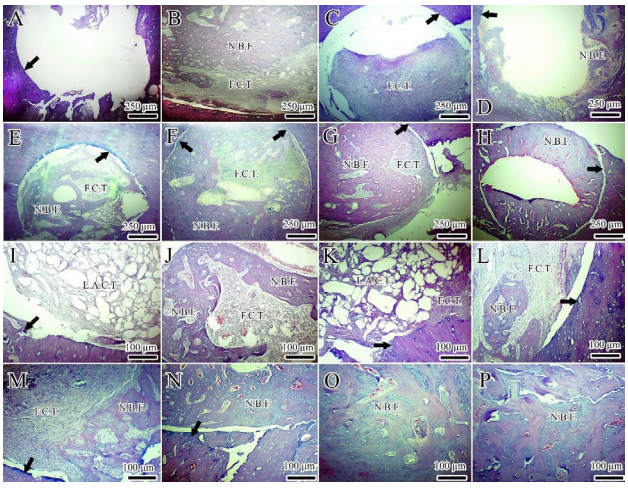

Figure 4. Histopathologic sections of the injured healing bone holes after 30 days of bone injury (Part I)
A&I: Untreated group. B&J: Autograft group. C&K: Gel group. D&L: Chi group. E&M: Hydroxyapatite group. F&N: nanohydroxyapatite group. G&O: Three calcium phosphate group. H&P: Strontium carbonate group. Arrows show the intact cortical bone. Abbreviations: N.B.F., New Bone Formation; L.A.C.T., Loose Areolar Connective Tissue; F.C.T., Fibrous Connective Tissue. Stained with H&E. Note that in the untreated group only a L.A.C.T. filled the hole and in the Gel and Chi groups a variable tissue consisting of mainly L.A.C.T. and F.C.T. filled the holes. In the Autograft group, a non-homogenous tissue consisting of F.C.T. and N.B.F. filled the hole. The most N.B.F. was observed in the TCP, Sr, nHA and HA, respectively. Gel: Gelatin, Chi: Chitosan, HA: Hydroxyapatite, nHA: Nanohydroxyapatite, TCP: Three calcium phosphate, Sr: Strontium

Thirty days after bone injury, only a loose areolar connective tissue similar to fascia formed in the bone holes that were either left untreated or treated with Gel. In the Chi group, micro-remnants of the Chi were observed that were phagocytized by the inflammatory cells. In the Chi group, also a few evidences of new bone formation were observed near the intact area of the bone at marginal area of the bone holes. The degraded Chi was replaced by the newly regenerated fibrous connective tissue consisting of fibroblasts that laid along the direction of longitudinally oriented collagen fibers. In the HA group, the HA powder was completely degraded and replaced by a combination of fibrous connective tissue and new bone that was a kind of woven bone with some immature primary osteons that were forming inside the holes. In the nHA-treated holes, the amount of fibrous connective tissue was lower than the HA group and more new bone formation was observed so that most of the hole was filled with new bone. In this new bone, several osteons were observed but they were immature and their lamellar bone had low diameter, while their haversian canal was large. Cortical bone that was remodeled from a woven bone was mostly observed in the lesions that were treated with either TCP or Sr. Thus, the most mature type of new bone was observed in these two latter groups. In these groups, secondary osteons were forming with many osteoblasts that were entrapped within their lacuna and transformed into osteocytes. Compared to TCP, in the Sr group, the osteons had larger transverse diameter with higher number of osteocytes within the circumferential lamella. However, in the TCP the new bone formation totally filled the holes and connected the intact bones with each other, while in the Sr group some of the bone holes were not completely filled with new bone (Figure 4 and Figure 5).

Scanning electron microscopy

The bone samples were evaluated by morphology and EDX analyses. In the EDX method, the peaks were

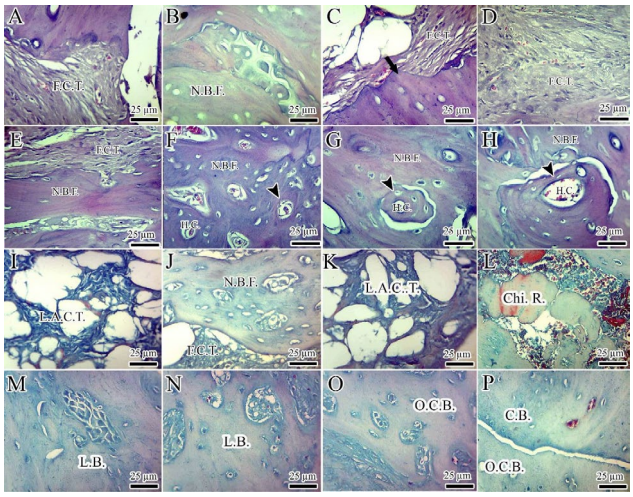

Figure 5. Histopathologic sections of the injured healing bone holes after 30 days of bone injury (Part B)
A&I: Untreated group. B&J: Autograft group. C&K: Gel group. D&L: Chi group. E&M: Hydroxyapatite group. F&N: nanohydroxyapatite group. G&O: Three calcium phosphate group. H&P: Strontium carbonate group. Arrow shows the intact cortical bone. Arrows head shows newly regenerated osteons. Abbreviations: N.B.F., New Bone Formation; L.A.C.T., Loose Areolar Connective Tissue; F.C.T., Fibrous Connective Tissue; Chi. R., Chi Remnant; L.B., Lamellar Bone; C.B., Cortical Bone; O.C.B., Old Cortical Bone; H.C., Haversian Canal. Stained with H&E (A-H) and Masson three chrome (I-P). Note that the untreated and also the treated bone holes with Gel were filled with L.A.C.T. In the Chi group, the holes were mainly filled with F.C.T. and the Chi. R. are observed that is present even after 30 days of injury. N.B.F. was obvious in those holes that were treated with ceramic materials. In comparison with HA, nHA, and TCP, largest osteons were regenerated in response to strontium carbonate. Gel: Gelatin, Chi: Chitosan, HA: Hydroxyapatite, nHA: Nanohydroxyapatite, TCP: Three calcium phosphate

visually defined as low, moderate and high. Based on the morphology and the peaks relative to calcium-phosphorous, different structures were distinguished from each other. In the untreated group and also in the Gel group only, the immature collagen fibrils were observed. At EDX analyses, few peaks relative to calcium-phosphorous were detected under SEM in these groups. In the Chi group, based on the SEM morphology and EDX analyses a new tissue varying from fibrocartilage to woven bone was observed so that the packed collagen fibrils were observed with moderate peak relative to calcium-phosphorous crystals. In the HA group, the morphology of the tissue showed a woven bone structure in which the primary osteons with large haversian canals were forming. In the nHA group, the primary osteons formed at that stage and high peak relative to calcification was detected only at areas of osteon formation but was moderate at intermediate spaces (just between the osteons). In the TCP and Sr groups, the secondary and the most mature type of osteons were observed that showed high peaks relative to calcification not only at the circumferential lamella (which is present in the osteons) but also between the osteons (intermediate lamella). In the autograft group, variable EDX peak relative to calcium-phosphorous were observed and the primary osteons were forming with the fibrocartilage that regenerated around the osteons (Figure 6).

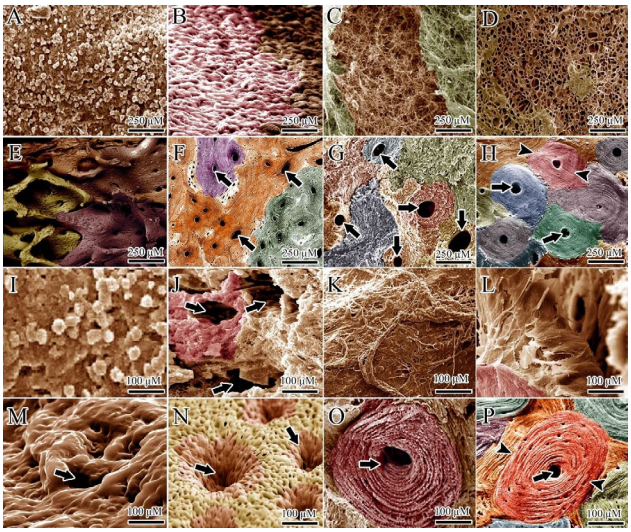

Figure 6. Scanning electron micrographs of the injured healing bones after 30 days of bone injury
A&I: Untreated group. B&J: Autograft group. C&K: Gel group. D&L: Chi group. E&M: Hydroxyapatite group. F&N: nanohydroxyapatite group. G&O: Three calcium phosphate group. H&P: Strontium carbonate group. Arrows show haversian canal and the arrows head show newly regenerated osteons. Note that the Gel and Chi failed to regenerate new osteons in the bone holes. The most mature type of new osteons was observed in response to strontium carbonate, three calcium phosphate, nanohydroxyapatite, autograft and hydroxyapatite, respectively. Gel: Gelatin, Chi: Chitosan

Dry matter content and bone ash

Thirty days after bone injury, the HA, nHA, TCP, and Sr showed significantly higher dry matter content and bone ash when compared to those of the untreated and also the Gel and Chi groups (P=0.001 for all). At this stage, the HA and nHA also showed no significant differences in term of dry matter content compared to autograft (P>0.05). Although HA showed significantly less bone ash than the autograft group, the nHA showed significantly higher bone ash compared to autograft (P=0.001 for both the comparisons). The TCP and Sr groups showed significantly higher dry matter content and bone ash compared to HA, nHA and autograft groups (P=0.001 for all the comparisons). At this stage, all the untreated, autograft, Gel, Chi, HA, nHA and TCP groups showed significantly less dry matter content and bone ash compared to intact normal bone (P=0.001 for all); however, there were no significant differences between Sr and normal bone in terms of dry matter content and bone ash at this stage (P>0.05) (Figure 7).

Bone densitometry

Thirty days after bone injury, the Gel group showed no significant difference compared to untreated group in term of BMD. Although the Chi showed significantly higher BMD compared to untreated group (P=0.001), it showed no significant differences compared to Gel (P>0.05), and both the Gel and Chi had significantly inferior BMD when compared to autograft, HA, nHA, TCP, Sr and normal groups (P=0.001 for all). There was no significant difference between autograft and HA in term of BMD (P>0.05) and both the autograft and HA

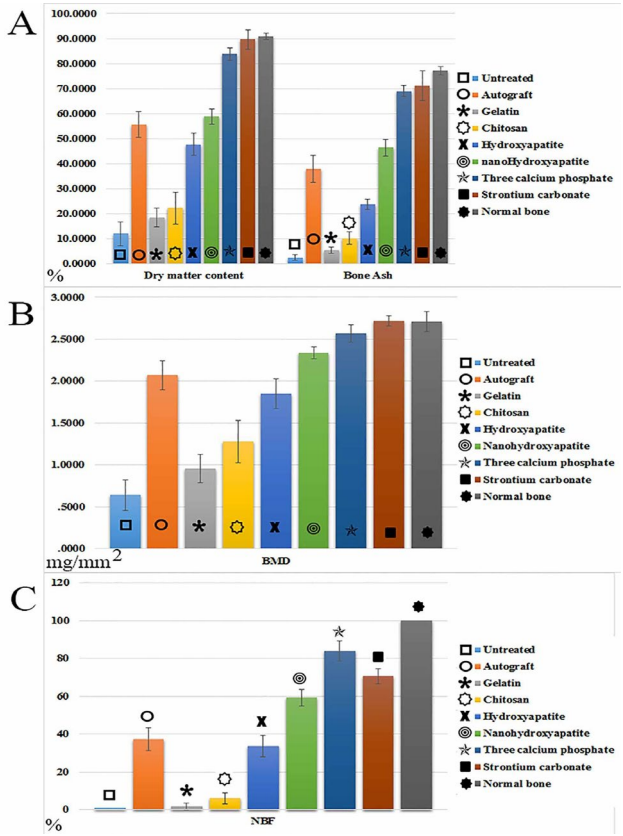

Figure 7. Percentage of dry matter content and bone ash, bone mineral density (measured by bone densitometry) and percentage of new bone formation (measured at histopathology) of the bone holes that were either left untreated or treated with various biomaterials; thirty days after bone injury

showed significantly lower BMD compared to nHA, TCP and Sr groups (P=0.001 for all). Both the TCP and Sr were superior to nHA in term of BMD (P=0.001 for both) and there were no significant differences between TCP and Sr and also between Sr and TCP with normal bone (P>0.05) at this stage (Figure 7).

Correlations of the findings

The optical density extracted from MTT assay at 48 hr after cell exposure showed positive correlation with the number of osteoblasts, osteocytes and also osteons measured at histopathology for the Sr group (r=0.595, P=0.021; r=0.634, P=0.009; r=0.578; P=0.032). In addition, there were a positive correlation between the optical density measured at 48 hr after cell exposure with that of BMD in the HA, TCP and Sr groups (r=0.612, P=0.007; r=0.733, P=0.005; r=0.752; P=0.001, respectively).

Discussion

This study showed that various biomaterials have different *in vitro* effects on cell viability and toxicity and also different *in vivo* effects in terms of tissue conduction, osteoconduction, osteoinduction and osteogenesis. Both the Gel and Chi showed few cytotoxicity, while nHA had no hazardous or toxic effects on cell viability and all the HA, TCP and Sr showed beneficial effects on cell viability, *in vitro*. This was probably due to the different shape and size of the materials particle that was shown by SEM images and was also probably due to their chemical interactions with ADSCs. At *in vivo* experiments, both the Gel and Chi showed no considerable osteoconduction, osteoinduction and osteogenesis and actually had no considerable role on bone regeneration. In contrast, ceramic materials including HA, nHA, TCP and Sr showed variable effects on osteoconduction and osteoinduction. Particle size had significant role on osteoinductive properties of the biomaterials so that the nHA showed superior osteoinduction and new bone formation compared to HA. In contrast to HA and nHA, TCP showed superior results in terms of osteoconduction and osteoinduction, while Sr showed the highest role on osteoinduction and formation of the most mature type of secondary osteons.

In the *in vitro* experiments, we evaluated cell viability and cytotoxicity of the biomaterials by MTT assay. In this method, we did not use carcinoma cells that have excellent proliferative properties because during normal bone healing, regional mesenchymal cells are a kind of stem cells that should ideally differentiate into chondroblasts and osteoblasts (1, 6). Thus, we used ADSCs. One of the other merits that we used ADSCs was that such cell type is routinely used to seed over and into the bone scaffolds, and then the cell-construct is implanted in the bone defects, *in vivo* (31-34). Thus, application of ADSCs for determining the role of different biomaterials on cell toxicity and viability has a great translational aspect (33). On the other hand, because all of the Gel, Chi, HA, nHA, TCP and Sr have been extensively used *in vitro* and *in vivo* with beneficial outcome on cell proliferation, differentiation, attachment and matrix production, and regarding that most of the studies have suggested their optimum cell cytocompatibility, we decided to neglect the indirect method in which the fraction of a suspended biomaterial is used for cell exposure, and directly applied the biomaterials on ADSCs (1, 9, 10, 12, 14, 16-20, 22). Our *in vitro* data suggest that all of the biomaterials that we used are highly cytocompatible; however, their degree of cytocompatibility is different. At the next step, the ultrastructure of the biomaterials was characterized by SEM. This let us know that the biomaterials are different based on the shape, size and surface, and this is possibly at least in part responsible for many of their *in vitro* and *in vivo* behaviors (1, 8).

In order to evaluate the role of various biomaterials on osteoconduction, osteoinduction and osteogenesis, we directly applied the Gel, Chi, HA, nHA, TCP and Sr on experimentally-induced bone hole model in rats as an established method for evaluating biomaterial powders (1, 6). Although we designed 5 femurs in each group, in the best situation the comparisons showed the differences between the animals. We decided to increase the number of bone holes in each femur from one to five holes in order to be able to compare the role of a particular biomaterial on a large number of bone holes between and within animals. This increased the reliability of the study. Because a 3 mm bone hole model was experimentally induced via surgical approach, and the bone holes were small enough to repair fast, we decided to evaluate the healing of injured bone samples after 30 days of bone injury. This time point let

us to observe significant differences between various biomaterials during bone regeneration (6). We evaluated the bone samples with different methodologies. We used routine histopathology and histomorphometry to semi-quantitatively count the cellular structures and osteons and also SEM to study the ultrastructure of the samples with aiding the EDX technology, which let to define the chemical composition of the area of interest under the SEM. In histopathology, the number of fibroblasts and fibrocytes were determined as an index of fibrous connective tissue formation, chondroblasts and chondrocytes as an index of hyaline cartilage formation, a combination of fibroblasts and fibrocytes together with chondroblasts and chondrocytes as an index of fibrocartilage formation, osteoblasts and osteocytes as an index of new bone formation, neutrophils as an index of inflammatory reaction, lymphocytes and macrophages as an index of chronic inflammation, osteoclasts as an index of bone remodeling, and finally primary and secondary osteons as indices of woven bone and compact bone formation, respectively (1, 6). The matrix components of the bone samples were evaluated histopathologically using double staining system and were confirmed by SEM and EDX analyses. The findings of morphologic and ultrastructural studies were further tested and confirmed by measuring BMD, dry matter content and bone ash. In fact, all of these methods can act as control and increase the reliability of the study. We measured dry matter content to measure the percentage of the organic and inorganic compounds of the samples. We also measured bone ash to directly measure the percentage of inorganic compounds of the samples (6). By subtraction of bone ash from dry matter content, the inorganic content of the samples was calculated. Measuring the BMD is another confirmatory method of SEM-EDX analysis and also the bone ash.

Thirty days after application of the Gel in bone holes, most of the defects were empty and only a loose areolar connective tissue filled the holes, so that the Gel was only a tissue conductive agent and was diagnosed as an inert biomaterial because neither hazardous nor beneficial effects were observed in this group. In contrast, Chi had more role on fibrous connective tissue formation, so that the new tissue that replaced the Chi was more mature than that of the Gel-treated samples; however, in both of the groups fibroblasts and longitudinally oriented collagen fibrils and fibers resembling scar tissue formation filled the bone holes. In *in vitro* experiments, Gel had higher cell cytotoxicity than Chi, while Chi showed less biocompatibility than Gel *in vivo*. These findings are not in line with those that showed *in vivo* biocompatibility of the Chi in chick embryos (35). This controversy could potentially be due to the molecular weight and size of Chi. In the chick embryos model, low molecular weight Chi was used and the Chi particles had nanostructured scale, while in our experiment we used medium molecular weight micro-structured Chi. Thus, we suggest that the molecular weight and size of Chi may significantly affect its biocompatibility *in vivo*.

The ceramic biomaterials showed totally different effectiveness during bone regeneration. The most important factor that must be addressed to achieve a successful clinical outcome is the biological compatibility of the material used to construct the implant or scaffold (15). Ceramic materials should not induce any cytotoxicity, immunological reactions, and inflammatory responses from the body (15). Based on the results of the present investigation, all the HA, nHA, TCP and Sr were highly cytocompatible and biocompatible and did not induce marked inflammation *in vivo*. HA considerably increased new bone formation; however, it failed to completely fill the bone holes with new bone and the fibrous connective tissue was present in the holes suggesting that HA increases regeneration of bone and also repair of the scar tissue. This finding is partially in line with those suggested that HA provides good osteoconductivity and offers good capabilities for osteoinductivity (36, 37). Based on our results, HA had good osteoconduction, while it had minimum value for osteoinduction when compared to other ceramics. It has been suggested that by changing the HA size, the osteoinductive properties of HA can be improved (15). When the size decreased, the repair properties of the HA significantly decreased and its regenerative properties significantly increased, so that in the nHA group the amount of scar tissue was too low, while most of the bone holes were filled with new bone. Although the nHA significantly improved osteoconduction and osteoinduction, its osteoinductive properties were not optimum because in the nHA group most of the osteons were still forming and the bone was developing from a woven bone. The major application of nHA in bone tissue engineering is to enhance bone implant surfaces. In a study, thin radio-frequency magnetron sputter deposited nHA films were prepared on the surface of a Fe-tricalcium phosphate (Fe-TCP) bio-ceramic composite showing that nHA improves surface wettability, adhesion and proliferation of mesenchymal stem cells *in vitro* (38).

In contrast to HA and nHA, the TCP showed significantly superior results in terms of osteoconduction and osteoinduction; however, repair capacity (scar tissue formation) in the TCP group was still evident. In the TCP group, the new bone almost completely connected the bone edges of the holes with new bone that was differentiating into compact bone because it had both primary and secondary osteons with corresponding circumferential and intermediate lamella. Because both the TCP and HA were effective during bone regeneration, their biphasic form may be an attractive option for increasing new bone formation. Osteoconductive potential and bone-healing pattern of biphasic calcium phosphates (HA-TCP) with varying compositions have recently been investigated showing that the specific HA-β-TCP ratios did not significantly influence new bone formation *in vivo* (39). In addition, in a clinical study it was shown that combination of platelet-derived growth factor and β-TCP resulted in a significantly higher BMD and new bone formation compared to HA + β-TCP (40). In another study, HA-β-TCP (30/70) increased new bone formation to about 30% in the patients, which was histomorphometrically superior to the patients receiving an organic bovine bone, mineralized solvent-dehydrated bone allograft, and equine bone, after a healing period of 6 months (41).

The different effectiveness of TCP and HA may

initially be due to their ultrastructural properties, which is due to their different chemical formulations (TCP: $Ca_3(PO4)_2$ vs. HA: $Ca_5(PO4)_3(OH)$). Based on the SEM studies we performed in this study, HA particles were round and amorphous, while the TCP particles had star like structures suggesting that chemically, TCP better interacts with the cells and molecules compared to HA and this may be the reason that most of the studies have suggested that HA has lower biodegradability than TCP (42, 43). In *in vitro* experiments and in line with our *in vivo* findings, it has been shown that TCP when incubated with rat bone marrow mesenchymal stem cells in a osteogenic culture medium has good cytocompatibility and increases the mRNA levels of various osteogenesis-related genes, including ALP, Rux2, COL-I, and SP7 (44).

In contrast to HA, nHA, and TCP, Sr showed the most superior results in term of osteoinduction because it considerably increased the size of the secondary osteons and this was responsible for the superior BMD and bone ash of this group. However, Sr showed moderate osteoconduction, so that in many cases it failed to completely close the bone holes. In line with our results, it has been shown that surface Sr modification by wet chemical treatment is a promising approach to enhance the early bone healing capacity of osteoconductive ceramic bone substitutes (45). By knowing that Sr has excellent osteoinductive properties and regarding that we showed it has moderate to low osteoconduction at *in vivo* level, combination of Sr with nHA or TCP would be an attractive option and this is why Sr is routinely combined with biphasic calcium phosphates in scaffold design and fabrication. In an experimental study in sheep, nHA-Sr has been shown to significantly increase bone volume compared to HA after 30 days (46). Sr can also be combined with other osteoinductive biomaterials. Recently it has been shown that Sr-substituted bioactive glasses significantly promote osteogenic responses of MC3T3-E1 osteoblast-like cells and inhibit the growth of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (47). In an *in vivo* investigation, it has also been shown that Sr-loaded samples accelerated the formation of new bone in both osteoporosis and bone defect models, as confirmed by X-ray, Micro-CT evaluation, and histomorphometric analysis of rats implanted with titanium nanotubes-Ag (22).

To the knowledge of the authors, this is the first to report the comparative effectiveness of various biomaterials on bone osteoconduction, osteoinduction and osteogenesis. We clearly showed their effects on cell toxicity, compatibility and viability *in vitro* and also on osteoconduction and osteoinduction, *in vivo*. Future studies should address how these biomaterials regulate bone signaling cascades and which formulation (combination of different biomaterials for scaffold fabrication) is able to exert the maximum effectiveness during bone regeneration and reduce bone repair by inhibiting scar formation.

Conclusion

Both the Gel and Chi showed some cytotoxic effects *in vitro*; however, Chi showed slightly higher inflammatory reaction compared to Gel *in vivo*. In addition, both the Gel and Chi had no significant role during osteogenesis and should only be used as a basic material for scaffold fabrication. The *in vivo* healing efficacy of such scaffolds should further be improved by embedding inorganic compounds particularly TCP and Sr. In contrast to Gel and Chi, the inorganic materials that we used in the present experiment showed superior cytocompatibility *in vitro* and biocompatibility *in vivo*. We showed that by reducing the size of biomaterial particles it is possible to enhance both of its *in vitro* and *in vivo* effectiveness, so that the nHA was found to be osteoinductive compound, while the HA was only able to improve osteoconduction. However, the nHA had less cell viability effects compared to HA. We also showed that different ceramic materials have different *in vivo* efficacy, so that TCP was found to be superior than both the HA and nHA in terms of osteoconduction, osteoinduction and osteogenesis. In contrast to TCP that had optimum role during osteoconduction, Sr showed the most osteoinduction with less osteoconduction. These data may be valuable for the scaffold designers in order to fabricate the most effective scaffolds for bone tissue engineering applications.

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Conflicts of Interest

This is the authors' own work. The authors declare that no conflict of interest exists.

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